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Hydrophobic Amino Acid Residues in the Acceptor Binding Site Are Main Determinants for Reaction Mechanism and Specificity of Cyclodextrin-glycosyltransferase*

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Cyclodextrin-glycosyltransferases (CGTases) (EC 2.4.1.19) preferably catalyze transglycosylation reactions with glucosyl residues as acceptor, whereas the homologous α-amylases catalyze hydrolysis reactions using water as acceptor. This difference in reaction specificity is most likely caused by the acceptor binding site. To investigate this in detail we altered the acceptor site residues Lys-232, Phe-183, Phe-259, and Glu-264 of *Bacillus circulans* strain 251 CGTase using site-directed mutagenesis. Lys-232 is of general importance for catalysis, which appears to result mainly from stabilization of the conformation of the loop containing the catalytic nucleophile Asp-229 and His-233, a residue that has been implied in transition state stabilization. Glu-264 contributes to the disproportionation reaction only, where it is involved in initial binding of the (maltose) acceptor. Phe-183 and Phe-259 play important and distinct roles in the transglycosylation reactions catalyzed by CGTase. Mutation of Phe-183 affects especially the cyclization and coupling reactions, whereas Phe-259 is most important for the cyclization and disproportionation reactions. Moreover, the hydrophobisity of Phe-183 and Phe-259 limits the hydrolyzing activity of the enzyme. Hydrolysis can be enhanced by making these residues more polar, which concomitantly results in a lower transglycosylation activity. A double mutant was constructed that yielded an enzyme preferring hydrolysis over cyclization (15:1), whereas the wild type favors cyclization over hydrolysis (90:1).

Cyclodextrin-glycosyltransferases (CGTase)[1] (EC 2.4.1.19) belong to the α -amylase family (glycosyl hydrolase family 13) (1), an important group of starch-converting enzymes. Catalysis in the α -amylase family proceeds via a covalently linked intermediate (2), which basically divides the reaction in two steps. In the first step the donor substrate (starch or oligosaccharide) is processed, yielding the covalent intermediate (donor reaction). In the second step the acceptor reacts with this intermediate, resulting in product formation (acceptor reaction). Whereas α -amylases usually catalyze a hydrolysis reaction using water as acceptor, CGTases mainly catalyze transglycosylation reactions in which the acceptor is either the non-reducing end glucose of another oligosaccharide (disproportionation) or the non-reducing end glucose of the covalently linked oligosaccharide intermediate, resulting in formation of a cyclodextrin (cyclization). Also the reverse of the cyclization, in which a cyclodextrin is cleaved and transferred to an accepting oligosaccharide (coupling), is catalyzed by CGTase (3). The main determinants that cause the difference in reaction specificity between α -amylases and CGTases are thus likely to be found at the acceptor binding sites.

The crystal structures of the *Bacillus circulans* strain 251 CGTase in complex with an acarbose-derived maltononaose inhibitor (4, 5) and a maltononaose substrate (2) have revealed the nature of the acceptor site. In the maltononaose structures the glucose residue at subsite +1 has hydrogen-bonding interactions with His-233, similar to those in *Aspergillus oryzae* α -amylase complexed with an acarbose-derived maltohexaose inhibitor (6). At subsite +2 hydrogen-bonding interactions also occur in both enzymes (with Lys-232; CGTase numbering) (Fig. 1*a*). In CGTase the +2 sugar residue is, in addition, sandwiched between phenylalanines 183 and 259 (see Fig. 1). These residues are well conserved in CGTases but not in α -amylases, where only Leu-232 (equivalent to Phe-259 in CGTase) has hydrophobic interactions with this glucose residue (6). The two aromatic residues have been shown to be important for the cyclization reaction (7), but their specific functions have remained unclear. Finally, a +3 acceptor subsite has been identified in the CGTase from *Thermoanaerobacter thermosulfurogenes* strain EM1, in which Glu-265 (Glu-264 in the *B. circulans* CGTase) is involved (8) (see Fig. 1*a*).

The above structural studies have given insights in the binding mode of linear oligosaccharides. A crystal structure of the *B. circulans* strain 251 CGTase in complex with a γ -cyclodextrin (9) revealed specific differences between the binding mode of linear and cyclic compounds. The glucose residue bound at subsite +1 has similar interactions in both cases, but the bound cyclodextrin has no hydrogen-bonding interactions at subsites +2 and +3 (Fig. 1*b*). The hydrophobic interactions with Phe-

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[1] The abbreviations used are: CGTase, cyclodextrin-glycosyltransferase; PCR, polymerase chain reaction; EPS, 4-nitrophenyl-α-D-maltoheptaoside-4–6-*O*-ethylidene.

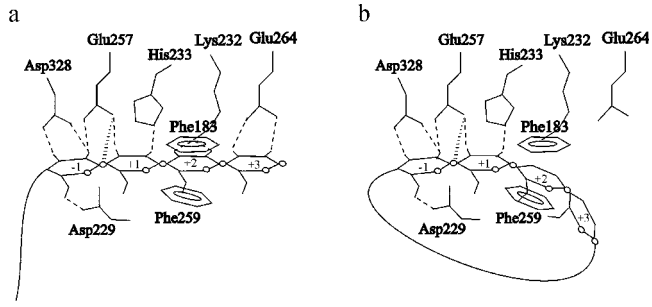

FIG. 1. **Schematic representation of the interactions between the *B. circulans* strain 251 CGTase and sugar residues bound in the active site.** *a*, binding mode of a linear oligosaccharide, representing binding of a donor substrate (2, 4, 8). *b*, binding mode of a cyclodextrin (9).

183 and Phe-259 are also different. Phe-183 has better stacking interactions with the linear compounds, whereas for Phe-259 these interactions are better with the cyclodextrin (Fig. 1).

These different interactions suggest that the residues involved may have different roles in the various reactions catalyzed by CGTase. We have investigated this by site-directed mutagenesis and detailed characterization of the mutant enzymes. Our study indeed shows that the various residues have distinct roles, dependent on whether disproportionation, cyclization, or coupling reactions take place.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Escherichia coli MC1061 (*hsd*R *mcr*B *ara*D139 Δ(*ara*ABC-*leu*)7679 Δ*lac*X74 *gal*U *gal*K *rps*L *thi*) (10) was used for recombinant DNA manipulations and site-directed mutagenesis. *E. coli* DH5α (F′/*end*A1 *hsd*R17 *sup*E44 *thi*1 *rec*A1 *gyr*A (Nal^r) *rel*A1 (*lac*ZYA-*arg*F) U196 (ø80d*lac*Δ(*lac*Z)M15) (11) was used for the production of monomeric supercoiled plasmid DNA for sequencing. CGTase (mutant) proteins were produced with the α-amylase- and protease-negative *Bacillus subtilis* strain DB104A (*amy npr*R2 *npr*E18 *apr*A3) (12). Plasmid pDP66K (13), with the *cgt* gene from *B. circulans* strain 251 under control of the p32 promoter (14), was used to introduce site-directed mutations and for production of the enzymes. Plasmid pBluescript KSII (Stratagene) was used for automated sequencing. DNA manipulations and calcium chloride transformation of *E. coli* strains were performed as described (15). Transformation of *B. subtilis* was performed according to Bron (16).

Site-directed Mutagenesis—Mutations were introduced with a PCR method using VENT-DNA polymerase (New England Biolabs, Beverly, MA) (13). A first PCR reaction was carried out with a mutagenesis primer for the coding strand plus a primer downstream on the template strand. The reaction product was subsequently used as primer in a second PCR reaction, together with a primer upstream on the coding strand. For the construction of Phe-183/Phe-259 double mutants the reaction product of the first PCR with the mutagenesis primer for Phe-259 mutations was used as primer in a second PCR reaction, together with the mutagenesis primer for Phe-183 mutations. This reaction product was subsequently used as primer in a third PCR reaction, together with a primer upstream on the coding strand. The products of the PCR reactions (1360 base pairs) were cut with *Pvu*II and *Sal*I, and the resulting fragments (1210 base pairs) were exchanged with the corresponding fragment from the vector pDP66K. The resulting (mutant) plasmids were transformed to *E. coli* MC1061 cells. The following oligonucleotide was used to construct the Lys-232 mutations: 5′-C ATG GAT GCA GTA C**W**G CAC ATG CCG-3′. The underlined cytosine is a replacement for the original thymine, resulting in removal of a *Nde*I site (CATATG). **W** can be an adenine or a thymine. An adenine results in the K232Q mutation; a thymine results in the K232L mutation and introduction of a *Sca*I site (AGTACT). For the construction of mutant E264A the primer 5′-C CTG GGC GTT AAC GCA GTG AGC CCG-3′ was used, resulting in introduction of a *Hpa*I site (GTTAAC). Oligonucleotides used to construct the phenylalanine mutations were as follows: for F183I, Thr, Asn, or Ser, 5′-GGC GGT ACC GAC A**N**T TCC ACG ACC-3′, containing a *Kpn*I site (GGTACC); for F259I, Thr, Asn, or Ser, 5′-GGC GAA TGG A**N**C CTA GGC GTA AAT GAA-3′, containing an *Avr*II site (CCTAGG). **N** can be any of the four bases, resulting in identical mutations with both primers. An adenine results in the Phe → Asn mutation, a cytosine in the Phe → Thr mutation, a guanine in the Phe → Ser mutation, and a thymine in the Phe → Ile mutation. The built in restriction sites allowed rapid screening of potential mutants. A mutation frequency close to 70% was observed; all mutations were confirmed by restriction analysis and DNA sequencing.

DNA Sequencing—Plasmids pDP66K carrying the correct restriction sites were cut with *Eco*RI and *Apa*I and with *Apa*I and *Sal*I. The resulting fragments were cloned in the multiple cloning site of plasmid pBluescript, and the resulting plasmids transformed to *E. coli* DH5α cells. Dideoxy sequencing reactions were done using T7 DNA polymerase, with either 5′-end-labeled primers or with unlabeled primers and fluorescein-labeled ATP (17, 18). Nucleotide sequencing was done with the automated laser fluorescent DNA sequencer (Amersham Pharmacia Biotech). The nucleotide sequence data were compiled and analyzed using the programs supplied in the PC/GENE software package (Intelligenetics).

Growth Conditions and Purification of CGTase Proteins—Plasmid-carrying bacterial strains were grown on LB agar in the presence of the antibiotic kanamycin at concentrations of 100 and 5 μg/ml for *E. coli* and *B. subtilis*, respectively (15). Agar plates also contained 1.5% potato starch (Sigma) for the detection of CGTase secretion by the bacteria. CGTase activity is visible on these plates by the precipitation of (β-) cyclodextrins, resulting in halo formation. *B. subtilis* strain DB104A with plasmid pDP66K, carrying wild type or mutant *cgt* genes, was grown for 24 h in a 2-liter fermentor, containing 1.5 liters of medium with 2% tryptone, 0.5% yeast extract, 1% sodium chloride, 1% casamino acids (pH 7.0) with 10 μg/ml erythromycin and 5 μg/ml kanamycin, to a final optical density at 600 nm of ~12. Under these conditions high extracellular CGTase levels were obtained reproducibly, allowing purification to homogeneity of up to 25 mg of CGTase protein per liter. The culture was centrifuged at 4 °C for 30 min at 10,000 × *g*. The (mutant) CGTases in the culture supernatants were further purified to homogeneity by affinity chromatography, using a 30-ml α-cyclodextrin-Sepharose-6FF column (Amersham Pharmacia Biotech) (19) with a maximal capacity of 3.5 mg of protein per ml. After washing with 10 mM sodium acetate buffer (pH 5.5), bound CGTase was eluted with the same buffer containing 10 mg/ml α-cyclodextrin.

Enzyme Assays—For all assays and enzyme dilutions a 10 mM citrate buffer (pH 6) was used. All incubations were carried out at 50 °C.

β-Cyclodextrin-forming activity was determined by incubating appropriately diluted enzyme (0.1–0.2 units of activity) for 2–4 min with a 5% solution of partially hydrolyzed potato starch with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands) preincubated at 50 °C for 10 min. At regular time intervals samples were taken, and the amount of β-cyclodextrin formed was determined based on its ability to form a stable colorless inclusion complex with phenolphthalein (20). One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin per min.

The coupling activity was determined as described by van der Veen *et al.* (3). The reaction mixtures containing cyclodextrin and methyl-α-D-glucopyranoside (66940; Fluka) were incubated for 10 min at 50 °C before the reaction was started with appropriately diluted CGTase. At regular time intervals (0.25 min) 100-μl samples were taken, and the CGTase was inactivated. The linear products were converted to glucose residues through the action of amyloglucosidase (EC 3.2.1.3, A-3514; Sigma). The glucose concentration was determined with the glucose/GOD-Perid method (124036; Roche Molecular Biochemicals). One unit of activity is defined as the amount of enzyme coupling 1 μmol of cyclodextrin to methyl-α-D-glucopyranoside per min.

The disproportionation activity was measured using the method according to van der Veen *et al.* (3). The reaction mixture contained up to 6 mM 4-nitrophenyl-α-D-maltoheptaoside-4–6-*O*-ethylidene (EPS, 1 492 977; Roche Molecular Biochemicals), a maltoheptasaccharide blocked at the non-reducing end and with a *para*-nitrophenyl group at its reducing end as donor and up to 10 mM maltose (Fluka 63418) as acceptor. After 10 min of preincubation at 50 °C the reaction was started with appropriately diluted CGTase. At regular time intervals (0.25 min) 100-μl samples were taken, and the CGTase was inactivated. Subsequently, the samples were incubated with α-glucosidase (EC 3.2.1.20, 1630385; Roche Molecular Biochemicals) to liberate *para*-nitrophenol from the product of the disproportionation reaction, non-blocked linear oligosaccharide. After addition of 1 ml of 1 M sodium carbonate the absorbance of the samples was measured at 401 nm ($\epsilon 401 = 18.4\ mM^{-1}$). One unit of activity was defined as the amount of enzyme converting 1 μmol of EPS per min.

The hydrolyzing activity was determined as described before (21). The hydrolysis of a 1% soluble starch (Lamers & Pleuger) solution, preincubated at 50 °C for 10 min, upon addition of CGTase was followed

TABLE I
β-Cyclization and hydrolyzing activities of wild type and mutant CGTases

(Mutant) enzyme	β-Cyclization	Starch hydrolysis
	units/mg	
Wild type	270.0 ± 1.7	3.2 ± 0.2
K232L	122.9 ± 6.5	ND[a]
K232Q	69.2 ± 3.5	ND
F183N	25.4 ± 1.7	10.6 ± 0.4
F183S	14.8 ± 1.2	8.8 ± 0.3
F259N	41.7 ± 0.2	60.3 ± 1.7
F259S	42.0 ± 1.8	32.6 ± 1.0
E264A	215.8 ± 11.9	ND
F183S/F259N	0.9 ± 0.1	14.3 ± 1.4

[a] ND, not determined.

by measuring the increase in reducing power (22). One unit of saccharifying activity was defined as the amount of enzyme producing 1 μmol of reducing sugar per min.

Kinetic analysis of the two substrate reactions (coupling and disproportionation) was performed with SigmaPlot (Jandel Scientific). The following equations (23) were used to fit the experimental data to determine which kinetic mechanism applies to the transglycosylation reactions catalyzed by CGTase the substituted-enzyme mechanism, represented by Equation 1:

$$v = V \cdot a \cdot b/(K_{MB} \cdot a + K_{MA} \cdot b + a \cdot b) \quad \text{(Eq. 1)}$$

the substituted-enzyme mechanism with substrate inhibition, represented by Equation 2:

$$v = V \cdot a \cdot b/(K_{MB} \cdot a + K_{MA} \cdot b \cdot (1 + b/K_{iB}) + a \cdot b) \quad \text{(Eq. 2)}$$

or through the ternary complex mechanism, represented by Equation 3:

$$v = V \cdot a \cdot b/(K'_{MA} \cdot K_{MB} + K_{MB} \cdot a + K_{MA} \cdot b + a \cdot b) \quad \text{(Eq. 3)}$$

where v is the reaction rate, V is the maximal reaction rate, a and b are the donor and acceptor substrate concentrations, respectively, A and B are the donor and acceptor substrates, respectively, and K_M and K'_M are the affinity constants for the substrates in the absence and presence of the second substrate, respectively. K'_{MB} is lost in the derivation of Equation 2, but it can easily be determined, because $K_{MA}/K_{MB} = K'_{MA}/K'_{MB}$.

Determination of Protein Concentration—Protein concentrations were determined with the Bradford method using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Screening and Identification of Mutant Enzymes—Several variants of residues 183, 232, 259, and 264 were obtained. A first screening on starch-containing agar plates revealed halo formation by all single mutants, indicating the production of β-cyclodextrin by the mutant CGTases. In contrast, several double mutants of residues 183 and 259 were incapable of halo formation. DNA sequencing of various selected clones resulted in the identification of the following CGTase mutants: F183N, F183S, K232L, K232Q, E264A, F259N, and F259S and the double mutant F183S/F259N.

Effects of the Mutations on the Cyclization Reaction—The β-cyclization activities of the wild type and mutant CGTase enzymes are shown in Table I. Changing hydrogen-bonding interactions at subsite +2 (Lys-232 mutants) clearly resulted in decreased cyclization activity, especially with mutant K232Q. Likewise, substitution of Phe-183 and Phe-259 by hydrophilic residues strongly reduced cyclization activity. Of the single mutants F183N and F183S showed the highest reduction in cyclization activity (10- and 20-fold). The double mutant F183S/F259N showed even a 300-fold decrease in cyclization activity, suggesting a synergistic action of the two phenylalanine residues. In contrast, in the subsite +3 mutant E264A the cyclization activity was hardly affected.

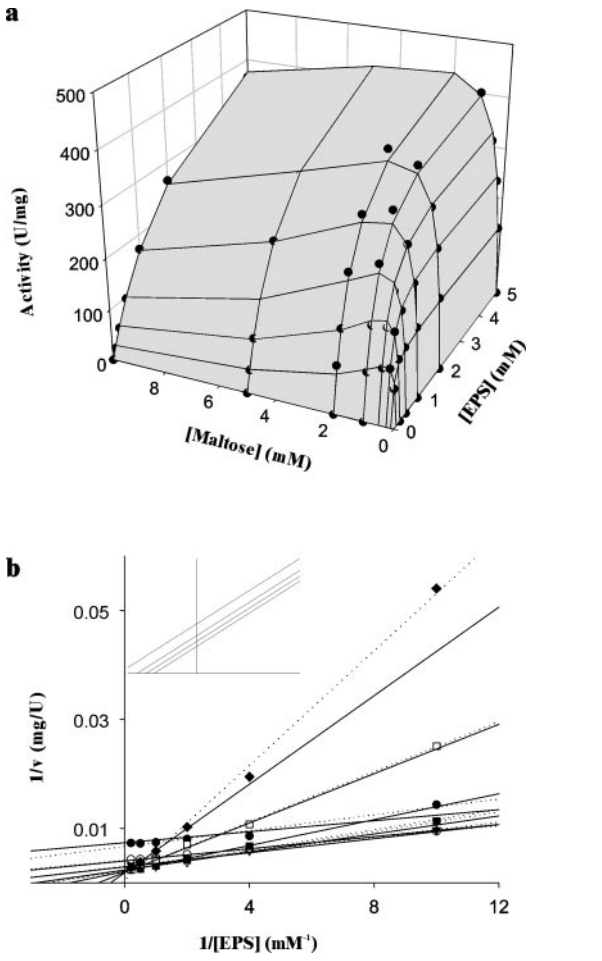

FIG. 2. **The disproportionation reaction of mutant F183N.** *a*, a mesh diagram showing the disproportionation activity of the mutant *versus* the substrate concentrations; substrate inhibition by maltose is clearly apparent. *b*, Lineweaver-Burke plot; the reciprocal of the specific activity (1/v) is plotted against the reciprocal of the EPS concentration at fixed maltose concentrations (in mM) (0.1, ●; 0.25, ○; 0.5, ▼; 1, ▽; 2, ■; 5, □; and 10, ♦). Linear regression results in the *dotted lines*, which clearly deviate from the *parallel lines* observed with the wild type enzyme (see *inset*). The calculated fit with the Sigma-Plot program using Equation 2 is represented by *solid lines*. *U*, units.

Effects of the Mutations on the Hydrolysis Reaction—The specific starch hydrolyzing activities of the (mutant) enzymes are listed in Table I. Especially the Phe-259 mutants showed a significant increase in hydrolysis (10- to 20-fold), whereas mutation of Phe-183 resulted in a 3-fold increase. Starch hydrolysis by the double mutant F183S/F259N was intermediate compared with the single F183S and F259N mutants.

Effects of the Mutations on the Disproportionation Reaction—For further characterization of the acceptor site, the disproportionation reaction was analyzed (see Fig. 2 and Table II). All mutations resulted in decreased disproportionation activities. For the Lys-232 mutants and mutant F259N this reduction in activity was comparable with that in the cyclization activity, suggesting similar roles for these residues in the two reactions. Mutations F183N and F183S, however, showed only 2- and 5-fold reductions in disproportionation activity (compared with 10- and 20-fold reductions in cyclization activity, respectively), suggesting that this residue has a more significant role in cyclization. As for the cyclization reaction, the double mutant F183S/F259N gave a much larger reduction in disproportionation activity (75-fold) than would be expected from a combination of the single mutants. The results thus suggest also a synergistic action of the two phenylalanine residues in the

TABLE II
Kinetic parameters of the disproportionation reaction of wild type and mutant CGTases

Mal, maltose.

(Mutant) enzyme	V_{max}	K_MEPS	K_MMal	K_iMal
	units/mg	*mM*	*mM*	*mM*
Wild type	970.0 ± 17.6	0.22 ± 0.02	0.83 ± 0.05	ND[a]
K232L	627.3 ± 15.6	0.29 ± 0.02	0.42 ± 0.03	4.7 ± 0.7
K232Q	303.3 ± 11.9	0.39 ± 0.04	0.29 ± 0.03	6.5 ± 1.5
F183N	556.3 ± 16.0	0.26 ± 0.02	0.31 ± 0.02	1.3 ± 0.2
F183S	170.3 ± 4.0	0.27 ± 0.03	0.27 ± 0.02	6.0 ± 1.3
F259N	189.2 ± 4.2	0.10 ± 0.01	0.14 ± 0.01	4.9 ± 1.1
E264A	520.6 ± 10.6	0.11 ± 0.01	0.10 ± 0.01	5.1 ± 1.1
F183S/F259N	13.7 ± 0.9	0.70 ± 0.06	0.01 ± 0.08	2.1 ± 0.3

[a] ND, not detectable.

TABLE III
Kinetic parameters of the β-coupling reaction of wild type and mutant CGTases

CD, cyclodextrin; ACC, acceptor.

(Mutant) enzyme	V_{max}	K_MCD	K_MACC	K'_MCD	K'_MACC
	units/mg	*mM*	*mM*	*mM*	*mM*
Wild type	294.0 ± 7.6	0.32 ± 0.02	18.1 ± 1.4	0.15 ± 0.04	8.5 ± 2.2
K232L	213.9 ± 15.6	0.47 ± 0.02	44.8 ± 4.3	0.35 ± 0.02	33.6 ± 4.3
K232Q	84.8 ± 15.6	0.49 ± 0.02	60.6 ± 4.3	0.32 ± 0.02	39.4 ± 4.3
F183N	1.6 ± 0.06	0.15 ± 0.02	6.2 ± 0.9	0.13 ± 0.07	5.1 ± 0.8
F183S	1.0 ± 0.03	0.41 ± 0.06	4.7 ± 0.6	0.43 ± 0.03	4.9 ± 0.5
F259N	117.0 ± 6.2	0.38 ± 0.04	17.7 ± 2.1	0.49 ± 0.05	22.6 ± 2.0
F259S	239.0 ± 15.2	0.54 ± 0.05	79.5 ± 7.1	0.29 ± 0.03	42.1 ± 4.0
E264A	282.7 ± 15.6	0.24 ± 0.02	17.8 ± 4.3	0.12 ± 0.02	8.7 ± 4.3
F183S/F259N	≪1	ND	ND	ND	ND

[a] ND, not determined.

disproportionation reaction. Finally, at subsite +3, mutant E264A showed a 2-fold reduction in disproportionation activity, indicating that this subsite, although somewhat remote from the catalytic site, contributes to this reaction. Surprisingly, all mutations, even the one at subsite +3, resulted in decreased K_m values for maltose and thus increased affinities. Furthermore, Fig. 2*a* clearly indicates substrate inhibition exerted by the acceptor substrate (maltose), which may be a direct effect of the increased affinities. Although the parallel lines in the Lineweaver-Burke plot for the wild type enzyme (see Fig. 2*b*, *inset*) indicate a normal ping-pong type of kinetics, represented by Equation 1, the experimental data of the mutants could best be fitted by Equation 2, resulting in the inhibition constants listed in Table II. The substrate inhibition by maltose was most pronounced for mutant F183N (shown in Fig. 2), although this mutant did not give the highest increase in affinity for maltose.

Effects of the Mutations on the Coupling Reaction—To delineate the roles of the acceptor site residues in the formation of the ternary complex in the coupling reaction (3), the effects of the mutations on β-cyclodextran coupling were analyzed (Table III). Drastically decreased coupling activities were observed with the Phe-183 mutants, suggesting an important role for this residue in the coupling reaction. The Phe-259 and Lys-232 mutants were more significantly affected in their substrate affinities, suggesting that these residues are more specifically involved in the formation of the ternary complex.

DISCUSSION

In the present study the +2 and +3 acceptor binding sites of CGTase were investigated in detail. Previously, structural and biochemical studies of CGTase had already provided evidence for the importance of His-233 in the +1 acceptor binding subsite for catalysis (24). Here we concentrate on subsites +2 and +3, more distant from the catalytic site. Our results show that Phe-183, Lys-232, and Phe-259, which interact with substrates and/or products at subsite +2, are involved in all CGTase catalyzed transglycosylation reactions, whereas Glu-264 at subsite +3 is only involved in the disproportionation reaction.

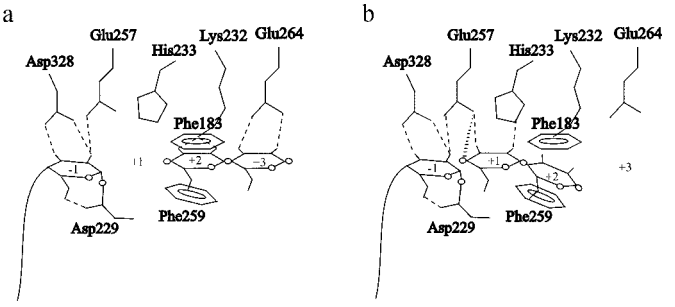

FIG. 3. **Schematic representation of acceptor binding at the active site of the *B. circulans* strain 251 CGTase.** *a*, initial "unproductive" binding at subsites +2 and +3. *b*, "productive" binding at subsites +1 and +2.

The Importance of Lys-232—Mutations in Lys-232 resulted in similar decreases in activity for all CGTase-catalyzed transglycosylation reactions, suggesting a general importance. Mutants K232Q and K232L have still appreciable activity, indicating that the polar headgroup of Lys-232 is not directly involved in catalysis. In contrast, reduction of the hydrophobisity of this residue (K232Q) results in more severely decreased activities than when the hydrophobic nature of the Lys-232 side chain is maintained (K232L). Lys-232 is situated in the loop following β-strand 3, which also contains the catalytic nucleophile Asp-229 and the His-233 residue. The apolar part of its aliphatic side chain has hydrophobic interactions with Trp-258, a residue completely conserved in CGTases that neighbors the acid/base catalyst Glu-257 in the loop following β-strand 4. Lys-232 thus appears to be important for structural integrity of the active site.

The Importance of Hydrophobic Residues at Subsite +2—Replacement of phenylalanines 183 and 259 by hydrophilic residues resulted in increased hydrolyzing activities and reduced transglycosylation activities. The physiological function of CGTase is to produce cyclodextrins and not short, linear oligosaccharides that can be utilized by competing organisms

in the natural environment. Therefore, hydrolysis of the substrate should be prevented, which CGTase accomplishes by excluding water from the active site and making use of an intricate induced-fit mechanism (25).[2] The introduction of hydrophilic residues near the catalytic site reduces the hydrophobic character of the active site and may favor the presence of water molecules that can react with the intermediate, as shown by the increased hydrolyzing activities of the Phe-183 and Phe-259 mutants. Indeed, combination of the two mutants even resulted in an enzyme that significantly prefers hydrolysis over cyclization (15:1), whereas the wild type favors cyclization over hydrolysis (90:1).

Phenylalanines 183 and 259 Have Distinct Roles in the Cyclization Reaction—The cyclization reaction requires a conformational change of the covalently linked oligosaccharide, producing a circular product from a linear substrate (circularization). Mutations of Phe-183 result in a larger decrease of the cyclization activity than those of Phe-259, suggesting a more important role for Phe-183. Crystal structures, however, show that Phe-259 has ideal stacking interactions with cyclodextrins at subsite +2 (9), whereas for Phe-183 these interactions are better with linear substrates (2) (see Fig. 1). Reaction path calculations on CGTase reconcile these apparently conflicting results (26). These calculations showed that Phe-183 is actively involved in the circularization process of linear oligosaccharides, whereas Phe-259 only comes into play at the end of this process, interacting with the non-reducing end sugars of the circularized covalent intermediate bound at the acceptor binding subsites. Our kinetic data on the mutant enzymes presented in this study substantiate now show for the first time that the two phenylalanine residues at subsite +2 affect the cyclization activity differentially. In addition, the loss of virtually all cyclization activity of the double F183S/F259N mutant supports an additive, consecutive action of the two subsite +2 phenylalanines.

Dual Binding Mode of the Acceptor Maltose in the Disproportionation Reaction—The disproportionation reaction operates via a ping-pong mechanism in which the processing of the first (donor) substrate is followed by binding of the second (acceptor) substrate (3). All mutations in the acceptor binding sites described here, including the E264A mutation at subsite +3, resulted in substrate inhibition exerted by maltose, whereas in the wild type enzyme no inhibitory effect of maltose on the binding of EPS is observed. This indicates that the CGTase active site is perfectly suited for the successive binding of donor and acceptor, in which subsite +3 also plays an important role. We propose that in the wild type enzyme a maltose can bind either at subsites +2 and +3 (Fig. 3*a*) or at +1 and +2 (Fig. 3*b*). The lack of substrate inhibition in the wild type enzyme, even at high maltose concentrations, indicates that the presence of the acceptor maltose does not affect binding of the donor EPS. The distribution of bound maltose over subsites +1/+2 and +2/+3 apparently facilitates exchange of this maltose for the donor substrate in such a way that no inhibitory effect of maltose is observed. Mutation of the acceptor site affects this distribution by changing the affinity for maltose, resulting in inhibition of the binding of the donor substrate by the acceptor substrate. This dual binding mode of maltose also explains the effects of the Phe-183 and Phe-259 mutations on the V_{max} of the disproportionation activity. Whereas for Phe-259 these effects are similar to those on the cyclization activity, for Phe-183 they are less pronounced and for F183N they are comparable with the effects of the E264A mutant. This suggests that Phe-183 is more specifically involved in the binding of maltose at subsites +2 and +3, which also involves interactions with Glu-264, whereas Phe-259 is more involved in the binding of maltose at subsites +1 and +2 required for catalysis. Thus, whereas Phe-183, together with Glu-264, provides an initial docking site for the acceptor maltose, Phe-259 serves to put the maltose in a (cyclodextrin-like) position suitable for catalysis. As for the cyclization reaction, this successive action of the subsite +2 phenylalanines explains why the double mutant F183S/F259N, in which both acceptor binding steps are affected, resulted in a very large decrease of the disproportionation activity.

The Role of the Acceptor Site in the Coupling Reaction—The kinetic mechanism of the coupling reaction proceeds via a ternary complex, in which the cyclodextrin (donor) and methyl-α-D-glucopyranoside (acceptor) bind simultaneously in the active site (3). Our results indicate that all mutated residues at subsite +2 affect the coupling reaction, whereas Glu-264 at subsite +3 does not. The data suggest that especially acceptor binding in the ternary complex is affected; for the Lys-232 mutants the affinity for the acceptor has decreased more than that for β-cyclodextrin, and for the Phe-183 mutants the coupling activity has decreased much more than the cyclization activity. Because the difference between the coupling and cyclization reaction is the formation of the ternary complex, this latter observation indicates the importance of Phe-183 for binding of the acceptor in the ternary complex, beside its role in the conformational change of the oligosaccharide (see above). In contrast, Phe-259 contributes to a lesser extent to the coupling reaction.

In conclusion, the hydrolyzing activity of CGTase is limited by the hydrophobicity of Phe-183 and Phe-259, as shown by the increased hydrolysis resulting from the replacement of these residues by hydrophilic ones. Furthermore, Phe-183 and Phe-259 have specific roles in the transglycosylation reactions catalyzed by CGTase. The current results support the action of these residues in the cyclization reaction as visualized by reaction path calculations; Phe-183 is most important for cyclization because of its intimate involvement in the 23Å relocation of the non-reducing end glucose of the covalently linked intermediate. In the disproportionation reaction, Phe-183, together with Glu-264, provides a docking site for the acceptor (maltose) at subsites +2 and +3. Thus, in both the disproportionation and the cyclization reactions, Phe-183 is involved in initial binding of the acceptor glucose residues. Phe-259, which specifically binds oligosaccharides in a "cyclodextrin" conformation, is required for final interactions resulting in catalysis.

By combining rational design and (semi) random mutagenesis we were able to select a double mutant (F183S/F259N) that significantly prefers hydrolysis over cyclization (15:1), whereas the wild type favors cyclization over hydrolysis (90:1). This shows that it is possible to specifically enhance one reaction over the other by site-directed mutagenesis of CGTase.

[2] H. Leemhuis, J. C. M. Uitdehaag, B. W. Dijkstra, and L. Dijkhuizen (2001) *J. Biol. Chem.*, submitted for publication.

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